AD NUMBER

AD287777

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; 01 OCT 1962. Other requests shall be referred to Chemical Corps Biological Lab., Fort Detrick, MD.

AUTHORITY

USADPG ltr, 7 Dec 1978

UNCLASSIFIED

AD 287 777

UNCLASSIFIED

287 777

First Quarterly Progress Report

Biochemical Detection Methods for
Bacteria and Viruses

Period Covered
1 July to 1 October 1962

Prepared by

M. A. Mitz
G. C. Blanchard

Contract DA 18-064 CML-2842
(J1042.01)

Submitted to

United States Army
Chemical Corps Biological Laboratories
Fort Detrick, Frederick, Maryland

Research Division
Melpar, Inc.
3000 Arlington Boulevard
Falls Church, Va.

UNCLASSIFIED

ASTIA AVAILABILITY NOTICE

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

ABSTRACT

The purpose of this work is to test the feasibility of biochemical methods for the rapid detection of airborne bacteria and viruses. Biochemical methods have distinct advantages for the development of detection methods for bacteria and viruses. The effort during the first quarter has been devoted to preparing a detailed work program and initiating the first phase of this experimental program. The experimental effort was directed at establishing that there are enzymes which are common to all the organisms tested and that the substrates employed were hydrolyzed at an appreciable rate.

We were surprisingly lucky in our selection of enzymes for study because the enzymes chosen, esterase and phosphatase, were detected on all thirteen organisms tested and were highly active and showed good rates even before any effort was made to optimize the conditions.

Also of interest was the finding that lyophilized cultures of Serratia marcescens, obtained from Dr. Marshowsky, the Project Officer, possessed about ten times as much esterase activity as the non-lyophilized Melpar stain of the culture. Of particular interest was the fact that the dehydrated organisms showed enzyme activity without a time lag.

The synthesis of several specially designed substrates to be employed in the next period of work was also accomplished during the first quarter.

1

1.   INTRODUCTION

This is the first quarterly progress report submitted in compliance with Contract DA-18-064-CML-2842 covering the work performed during period 1 July to 1 October, 1962. The contract effort is concerned with demonstrating the feasibility of using biochemical methods for the detection of bacteria and viruses. There are a number of biochemical approaches which might be applied to the detection problem including enzymatic amplification, chromophoric substrates, detection of cofactors, bioelectric means, spectrophotometric means and immunological means. Because all of these methods cannot be investigated with the funds available, a priority has been placed on the catalytic processes, i.e. enzyme amplification, chromophoric substrates, cofactors and bioelectric methods. The reason for choosing catalytic processes for initial investigation is based on the fact that the effect of these catalysts on specific substrates can be amplified directly by microscopic means or indirectly by using the enzymatic products to catalyze other enzyme systems.

The contract effort has been divided into three tasks: Task I covered the period 1 July to 30 September 1962; Task II covers the period 1 October to 31 December 1962; Task III will cover the period 1 January, through 30 June 1963.

Task I consisted of two efforts: (a) planning and design of the experimental program, procurement of materials and (b) testing of bacteria for the presence of a universal enzyme. Due to the late receipt of the contract, no money was spent during the month of July. Work was begun, however, on the collection and purification of microbial cultures to be

2

used in the program. Also during this period the methods proposed by Melpar were re-evaluated and refinements were added. The detailed outline of the program submitted for approval is shown in Appendix. The latter effort (b) was carried out between 15 August and 30 September 1962.

Task II will consist of two major efforts. One effort will be concerned with the testing of amplified enzyme systems. The second effort will be concerned with tests to determine the optimum conditions for esterase and phosphatase activity. Task III will consist of studies concerned with the quantitation of amplified methods, the testing of bioelectric techniques and certain chromophoric substrates. The last month of the program will be used for the preparation of the final report and for the selection and design of the best detection methods.

2.   EXPERIMENTAL WORK

2.1   Introduction

The effort for this first quarter was directed towards determining the esterase and phosphatase activity of twelve bacterial cultures and one yeast culture to obtain data relating to the following: (1) that esterases or phosphatases are common to all organisms and (2) that the substrates are hydrolized by these organisms at an appreciable rate.

The esterase and phosphatase enzymes were the first ones selected for a number of reasons. These enzymes appear to be essential in metabolism, and the making and breaking of ester bonds occurs universally in living cells. The substrates (esters) for these enzymes are numerous and easily defined

3

and the simpler ones are relatively easy to obtain either by purchase or synthesis. The assay methods for these enzymes are simple and easy to interpret. The hydrolysis of most esters can be monitored by acid production; for special esters there are individual sensitive methods such as spectrophotometry. The mechanism of ester hydrolysis can be presented as follows:

$$\underset{RC}{\overset{O}{\overset{||}{}}} - OR^1 + H_2O \underset{\longleftarrow}{\overset{catalyst}{\longrightarrow}} R - \overset{O}{\overset{||}{C}} - OH + R^1OH$$
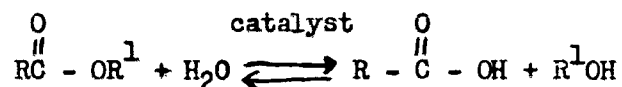
Note that the reaction is an equilibrium reaction and that it can proceed in either direction. The extent to which the reaction will go to the right, toward complete ester hydrolysis, will depend on the pH of the solution. In order to maintain a maximum rate in any equilibrium reaction, the products of the reaction must be removed from the system. In this case, the neutralization of the acid formed is enough to drive the reaction to the right.

$$R - \overset{O}{\overset{||}{C}} - OH + OH^- \rightleftharpoons R - \overset{O}{\overset{||}{C}} - O^- + H_2O$$
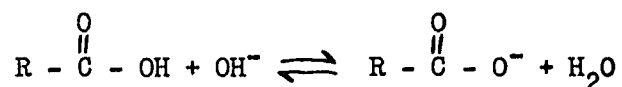
The above theoretical consideration for esterase can also be used for the phosphatases because the substrates for the phosphatases are esters of phosphoric acid. The only difference between simple organic esters and phosphate esters is the pKa of the acid liberated. The organic acid liberated is usually monobasic with a pKa of 3.5 and above, while the phosphoric acid is tribasic with pKa's at the very low, nearly neutral and very basic regions. Therefore, it is possible for phosphatase enzymes to have a pH optimum in the acid, neutral or alkaline range. Thus by buffering the enzyme assay system, one can determine acid or alkaline

phosphatases. The study reported herein was concerned only with the alkaline phosphatases, but the acid phosphatases may be examined at some future date.

It was our original intent to study non-pathogenic species of organisms occurring in genera in which interesting pathogens occurred. For example B. pumilus and B. globigii were chosen because they simulated B. anthracis. Pseudomonas stutzeri was chosen because it simulates P.aeruginosa. Saccharomyces cerevisiae was selected because it is a yeast which might simulate some of the pathogenic yeasts such as Cryptococcus neoformans, Candida albicans and Coccidioides immitis. S. marcescens, E. coli and P. vulgaris were chosen because they simulate some of the enteropathogenic organisms such as E. coli, Salmonella typhosa, Salmonella typhimurium, Salmonella enteritidis and others.

Some of the organisms such as Neisseria catarrhalis which simulates N. gonorrhea; Pasteurella anatapestifer which simulates P. pestis; Shigella flexneri which simulates S. dysenteriae; Corynebacterium pseudodiphtheriae which simulates P. diphtheriae; Clostridium autobutylicum which simulates Cl. botulinum; Vibrio metchnikovii which simulates Vibrio cholera and others are in the Melpar stock collection but failed to grow well under the growth conditions established for this study; thus, they were not considered at this time. Attempts to obtain other simulants for pathogens in the genera Hemophilus,Asterococcus, Actinobacillus, and Streptococcus and others will be continued. Also the testing of the present simulants for enzymes will be carried out where possible.

Some of the organisms tested for enzymes, for example
Azotobacter vinelandii, _Sarcina_ _lutea_, _Aerobacter aerogene_ are common
soil organisms often present in the air. These organisms were
useful in demonstrating that esterases and phosphatases are universally
present on microorganisms.

The conditions employed for the growth of each of the organisms
were chosen because some standardized reproducible set of conditions
were thought to be necessary. The medium was selected because it was
rich and would support the growth of most of the organisms. The length
of incubation was chosen because the cells are in their log place at
the time of harvest, thus the cells are in their most active state of
metabolism. Also the enzymes present in the bacteria should be at their
highest concentration during this period of growth.

The work accomplished and the conclusions obtained are
discussed in the next section.

2.2   Purification and Identification of Microbial Cultures

A great deal of time during the first month of the work was
spent on the isolation, purification and identification and maintenance
of pure cultures. Because cultures obtained from American Type Culture
Collection are often contaminated and because the validity of the
enzyme studies, relating to the universal enzymes, were dependent
upon pure cultures, the effort was thought to be well justified.
The purity of each culture was checked by several different procedures.
First, the culture was grown 12 - 18 hours in sterile trypticase soy
broth (described below). A sample of the broth was then serially diluted
in sterile saline (0.85% NaCl) and plated in trypticase soy agar. Also
a sample of the broth was streaked on an agar plate so that isolated
colonies could be found. After 12 - 18 hours incubation at the optimum
temperature for the growth of the organism, the gross morphology of the
isolated colonies were examined macroscopically. A microscopic examina-
tion was made from portions of the isolated colonies by stabbing the
colony with a sterile inoculating loop and preparing a gram stained smear
of the material. If the colonies were found to be pure microscopically,
they were streaked on a sterile agar slant and maintained pure. If the
colonies were still contaminated however, they were again inoculated into
broth and replated to obtain isolated colonies. In some instances it was
not possible to distinguish which were the contaminant colonies and thus
each of the colonies had to be subjected to a variety of biochemical tests.
Identifications were made on the basis of biochemical tests such as
growth on a variety of sugars, citrate, or on the ability of the culture
to produce indole, acetylmethyl carbinol or H_2S or by other distinguishing

7

reactions, characteristic for the culture of interest.

2.3   Growth and Quantitation of Microorganisms

The sequence used for obtaining organisms for enzyme assay
was as follows: (1) growth of the inoculum (2) growth of the
organisms on the surface of agar (3) harvesting and washing of the
cells (4) testing for enzyme activity and (5) numerical quantitation
of the organisms. The detailed procedure is described below. The
medium used for the growth of all of the microorganisms was trypticase
soy broth.[a] The agar was prepared by adding 15 grams of agar per liter
of broth. All media were sterilized by autoclaving before inoculation
with microorganisms. All of the microbial cultures used in the
experiments reported herein were pure cultures. The inoculum for the
spread plates was prepared by growing each of the organisms in broth for
12-18 hours at $30^{o}C$. One ml of inoculum was pipetted onto solid trypticase
soy agar previously poured into a petri plate. The inoculum was spread
evenly over the agar by employing a Fisher turntable for rotation of the
plate and a bent glass rod spreading the bacteria evenly over the surface
of the agar. The glass rod was sterilized by flaming afrer dipping in
alcohol. The plates were allowed to incubate at $30^{o}C$ for 16-18 hours
and the layer of bacteria was scraped from the agar with a sterile bent
spatula. The organisms were suspended and washed three times by centrifu-
gation, in sterile 0.85% NaCl. The microbial slurry was then diluted
in distilled water for microscopic counts, plate counts and enzyme assay.

---

[a]Purchased from Baltimore Biological Laboratory, Baltimore, Md. Prepared
as directed on bottle.

The microorganisms were assayed for enzyme activity within one hour of preparation. The microscopic count quantitated the total count of viable and nonviable organisms whereas the plate count quantitated only the viable organisms. A comparison of microscopic and plate counts is shown on Table 1. The microscopic counts were made in a Petroff-Hauser counting chamber. Only slight differences occurred between plate counts and microscopic counts. In the future, only microscopic counts will be used for quantitation of the microbial populations, except where greater precision relating to the viable microbes is required.

2.4   Enzyme Studies

The microorganisms were assayed for two different esterases, phenylacetate esterase and 2,6 dichloroindophenyl acetate esterase, and for alkaline phosphatase.

2.4.1   Phenylacetate Esterase

Procedure: The phenylacetate esterase assay was carried out in a Radiometer Autotitrator Model TTTI equipped with Beckman probe type microelectrodes with an extended range in the alkaline region. A 2 ml microburette calibrated in hundreds of ml was attached to a magnetic valve to deliver alkali as required by reaction. The electrodes and titrator were standardized against Beckman pH 7 buffer solution each day before performing the enzyme assays. The reaction mixture for the enzyme assay consisted of 2 ml of 0.2 M $MgCl_2$, 1 ml of phenylacetate,* 0.01M, and 1 ml of a 10^1 dilution of the microbial suspension. The reaction mixture was stirred constantly with a magnetic stirrer. The

_____
*Highly pure No. 258, Eastman Chemical Co., Rochester, N.Y.

actual number of organisms used to start the reaction is shown in Table 1. The enzyme reaction rate was determined by recording the amount of 10^{-3}M KOH required to maintain the hydrogen ion concentration of the reaction mixture constant at pH 7.4 during the ten minute incubation period at 20°C. The amount of KOH used during the assay was recorded every 30 seconds of the ten minute incubation.

Results: All the experiments were carried out in duplicate and the results present in all the Tables and Figures are mean values of these determinations. The variation between duplicates was not more than 5%. The rate that phenylacetate was hydrolyzed by each of the microorganisms is shown in Figures 1, 2 and 3.

Discussion of results: The ideal type of rate curve is shown in Figure 1, where Bacillus globigii hydrolyzed the substrate at a constant rate for most of the ten minute period. This is the type of curve that is expected for most enzyme reactions where inhibition or destruction of the enzyme is absent.

The lack of linearity in the curves in Figures 2 and 3 indicate that different amounts of substrate are hydrolized by the microroganisms at each of the time increments. There are several possible explanations which might be applied to these results. The lag period observed with S. aureus, S. marcescens and S. cerevisiae (Figure 2) might be due to (1) an artifact in the method of assay (2) a buffering property of the bacteria or (3) the lack of surface enzymes. Bacteria may have considerable buffering properties which would take some time to overcome and this would manifest itself as a period of no acid production. It may be possible to overcome this if the organisms are incubated with the buffer

before addition of the substrates. The lag period may also be associated with cell permeability; thus time would be necessary for diffusion of substrate into the cell and products out of the cell.

The rapid initial hydrolysis of phenylacetate by A. vinelandii, S. flexneri and P. vulgaris (Figure 2) and by B. alba, P. stutzeri, B. pumilus and A. hydrophila (Figure 3) for the first two minutes of the reaction followed by a decreased rate in hydrolysis might be the result of (1) a low, limiting substrate concentration (2) the build up of inhibitory end products or (3) the inhibition by increased monovalent cation (K+) concentration resulting from increased KOH in the reaction mixture. The first explanation does not appear to be valid because B. globigii (Figure 1) hydrolized five to 20 times as much substrate as the above cultures. Cation inhibition has been observed previously by Blanchard[1] with purified preparations of hexokinase, where reaction rate curves similar to those reported herein were obtained. This difficulty can be overcome by using basic organic compounds such as Tris (trishydroxymethylaminomethane) buffer.

When the conditions for optimum enzyme activity are established, it is expected that the amount of substrate hydrolized will be greatly increased. Optimum conditions are also expected to improve the enzyme reaction rates. Increased reaction rates and thus increased substrate hydrolysis will greatly improve the sensitivity of the assay procedures.

2.4.2   2,6 Dichloroindophenylacetate (IPA) Esterase

All of the bacteria were screened for esterase activity with another substrate, 2,6dichloroindophenylacetate (IPA)* This substrate

---

*Synthesized by Melpar's Organic Laboratories according to Procedures in Reference 13.

11

was readily hydrolized by the test system (serum esterase), but was
not hydrolized by any of the microbial enzymes. The same concentrations
of microorganisms were used in this assay as were used in the
phenylacetate esterase experiments (Table 2).

Procedure: Modification of Kramer and Gamson [13]

The stock substrate was prepared by dissolving 20 mg IPA
in 10 ml of methanol. This solution was not stable and therefore was
prepared fresh daily. The reaction mixture contained 0.1 ml of
IPA stock (0.63 u moles) 2.0 ml of 0.1 M Sorenson's phosphate buffer
pH 7.4, 3.0 ml distilled water and 1.0 ml of a dilution of the microbial
suspension. The reaction was followed on a Beckman DU spectrophotometer at a
wavelength of 613 mu. Substrate hydrolysis is indicated by increased
absorption of the liberated indophenol groups. The assay was carried out
in duplicate with three dilutions of the bacterial suspension.

Results: No substrate hydrolysis was observed with any of
the bacteria listed in Table 1, even after an hour of incubation.

Discussion of Results: Apparently the microbial enzyme is
quite specific because a control using horse serum esterase gave
appreciable hydrolysis of the substrate in two minutes. Similar specificity
has been found with other esterases. For example, serum esterase and liver
microsomal esterase hydrolyze two substrates, IPA and phenyl acetate,
but stroma esterase hydrolyzes only one of the substrates, phenyl acetate.
After optimum enzyme assay conditions are established this substrate will
be checked again. The lack of enzyme activity may also be associated

with inhibition caused by the methanol. This possibility can be
tested by adding different concentrations of methanol to the
reaction mixture for phenylacetate hydrolysis. .

2.4.3   Alkaline phosphatase

Alkaline phosphatase is another type of esterase which appears
to be universally present in all of the organisms tested. (Table 4).
The turnover number (number of molecules of substrate hydrolized per
organism in one minute) extrapolated from the whole incubation time
for this enzyme was slightly less than was observed for the phenyl-
acetate esterase. The same standard alkaline phosphatase conditions were
used for all organisms. It is expected that the optimized enzyme assay
conditions will greatly improve the turnover number.

Procedure:

The assay procedure used was a modification of the Bessey,
Lowry, Brock procedure[2] as modified by the Sigma Chemical Company in
their bulletin No. 104 (1958).

The principle of the test is that the substrate p-nitrophenyl
phosphate is colorless in acid or alkali but after hydrolysis forms p-
nitrophenol which is yellow in alkali and colorless in acid. The reaction
is: p-nitrophenyl phosphate + H_2O $\xrightarrow{\text{phosphatase}}$ p-nitrophenol + H_3PO_4

The stock substrate was prepared by dissolving 0.1 gm. p-nitro-
phenyl phosphate* in 25 ml. of distilled water. The reaction mixture con-
tained 0.5 ml of stock substrate, 0.5 ml of 0.1 M glycine buffer pH 10.5
and 0.1 ml of water (in reagent blank) or 0.1 ml of diluted bacterial sus-
pension. The 1.0 ml of buffered substrate was allowed to equilibrate in a
water bath at 38° for five minutes prior to the addition of the microbial
suspension. After addition of the microorganism the reaction mixture was
allowed to incubate at $38^\circ C$ for 30 minutes. The reaction was stopped after

* Disodium salt, K & K Laboratories, Jamaica, N.Y.

incubation by the addition of 10 ml of 0.02N sodium hydroxide. The contents in the 18 x 150 mm calibrated tubes used in the assay were then mixed by inversion. The absorption due to the liberated p-nitrophenol was compared to the reagent blank in the Coleman Universal spectrophotometer Model 14, at a wavelength of 400 mμ. The amount of p-nitrophenol (μ moles) liberated in the reaction was determined from a standard curve of p-nitrophenol. The standard curve (0.05 - 0.5 μ moles p-nitrophenol) was prepared by first preparing a 10 m M stock solution by dissolving 197.13 mg p-nitrophenol in 100 ml of distilled water. An aliquot of this stock solution (0.5 ml) was mixed with 99.5 ml of 0.02 N NaOH to give a final concentration of 0.05 μ moles/ml. Aliquots of this solution 1 - 10 ml were added to cuvettes and diluted to 11 ml with 0.02 N NaOH. The absorption was read on the spectrophotometer and the standard curve was prepared by plotting the results.

Discussion of results

The enzyme reaction rates were not followed for this enzyme. The results shown in Table 4 only establish that alkaline phosphatase is fairly universal in the microorganisms. In the future when the conditions for optimum enzyme activity are explored, the enzyme reaction rates will be followed, and more accurate turnover numbers will be calculated.

2.4.4   Enzyme activity of lyophilized cultures

Lyophilized preparations of Serratia marcescens and Bacillus glooigii, supplied by Dr. Warshowsky the Project Officer, were suspended in distilled water and tested for phenylacetate esterase activity. The total number of bacteria used in the esterase assay was determined by microscopic count and by plate count techniques (See Table 1). The esterase activity (turnover number) for the lyophilized preparation of S. marcescens was greater than was observed for the non-lyophilized culture from the

Melpar stock collection (See Table 3). No enzyme activity was observed for the B globigii culture. This lack of enzyme activity is probably due to the fact that the culture is not viable, possibly because of sterilization before shipment.

The difference observed in the activity of the two different strains and two different preparations of S. marcescens may be due to a difference in growth medium or a strain difference. The differences can be resolved by growth of the two different strains on the same medium prior to the enzyme assay.

The enzyme rate curves demonstrated by the two preparations of S. marcescens were also different. The lyophilized preparation gave rapid hydrolysis of the substrate for two minutes before leveling off (Fig.4). The non-lyophilized preparation had a one minute lag period and a nearly linear rate. The most surprising result however, is that the lyophilized culture has about ten times as much enzyme activity per cell as that observed with the non-lyophilized culture (See turnover Numbers Table 3). Although these results are not conclusive, they are encouraging because they indicate that lyophilization does not greatly diminish the activity of the esterase enzymes. The stability of the lyophilized culture and the effect of storage in the lyophilized state on the activity of the enzyme will be tested periodically throughout the contract period.

2.5   Substrate Synthesis

Several substrates to be used in the enzyme amplification experiments were synthesized by the organic chemistry laboratories of Melpar. There are two types of substrates which are to be used in the initial investigations; fluorescent substrates and esters of sulfhydryl compounds. The compounds synthesized to date are fluorescein mono-acetate, fluorescein diacetate and p-nitrothiophenyl acetate.

One of the most interesting fluorescent dyes investigated was 6-hydroxyfluoran. Rotman[3] reported that this compound had a strong fluorescence peak at 490 mμ while the substrate for B-galactosidase, 6-hydroxyfluoran - B - D - galactopyranoside, had its fluorescence peak at 520 mμ. This compound was reported to be better than fluorescein because when the free OH group is chemically bound, i.e. as the galactoside or acetate, no fluorescence is observed. Thus fluorescence occurs only after enzyme action. For this reason, attempts were made to synthesize this dye.

2.5.1   Attempted Synthesis of 6-hydroxyfluoran

Unsuccessful attempts were made to prepare 6-hydroxyfluoran according to the procedure of Ghatak and Dunn.[4] The above workers reported that they obtained the product, 6-hydroxyfluoran, when they reacted 2,4-dihydroxy-benzoyl-benzoic acid with phenol in the presence of H_2SO_4. The reaction is written below:

2,4 dihydroxy-benzoyl-benzoic acid

6-hydroxy fluoran m.p. 181°

17
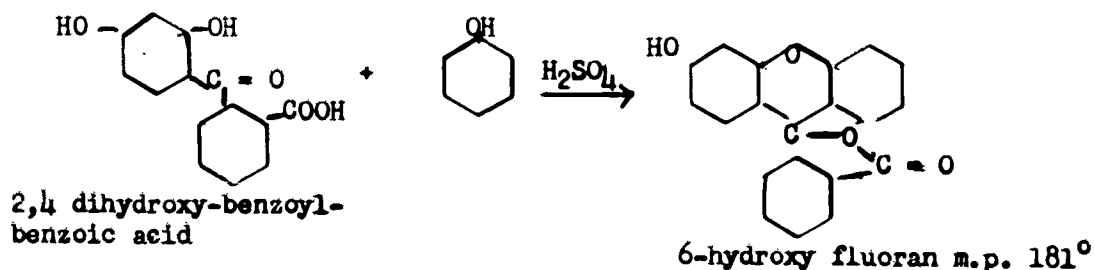
Melpar's organic laboratory synthesized 2,4-dihydroxy-benzoyl-benzoic acid (m.p. 212-214°C) from fluorescein by procedure of Ghatak and Dunn[4]. When this compound was reacted with phenol however, the product recovered was not 6-hydroxy-fluoran but instead appeared from melting point demonstrations to be fluorescein. A telephone conversation with Dr. Boris Rotman, Department of Genetics, Stanford University Medical School, Palo Alto, California revealed that they too had run into difficulties with the synthesis of 6-hydroxyfluoran. Dr. Rotman said that they were unable to obtain 6-hydroxyfluoran from the reaction, but instead obtained the starting material, fluorescein. He also said to disregard the comparisons made in his paper[3] regarding fluorescein and 6-hydroxy-fluoran because he found out too late that he was really working with fluorescein and fluorescein galactosides.

Because the 6-hydroxyfluoran dyes could not be synthesized and because Rotman was apparently working with fluorescein derivatives, it was decided that esters of fluorescein would be synthesized. Some of the most interesting esters, based on the universal enzymes are acetate and phosphate esters.

Fluorescent substrates from compounds other than fluorescein, for example, naphthyl fluorescein, salicylic acid, indoxyl etc. will be synthesized in the future as they are needed.

2.5.2   Synthesis of Fluorescein Monoacetate[5,6]
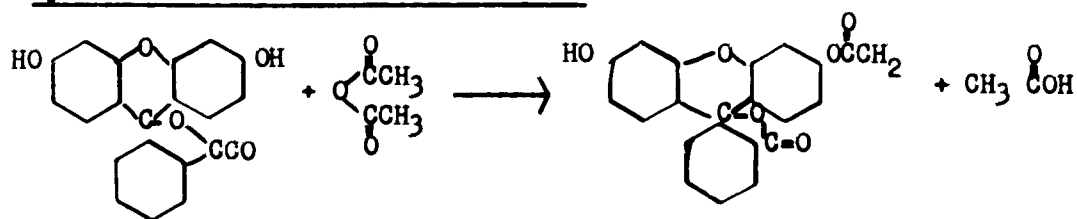
Procedure: In an Erlenmeyer flask dissolve 10 g. (.03 moles) of fluorescein in 15 cc of 3N NaOH and pour the solution over 30 g. of ice. Keeping the solution cool at all times in an ice bath, add carefully 3 g. (.03 moles) of acetic anhydride dropwise (with stirring) to the disodium fluorescein. Acidify the alkaline solution with 10% HCl and filter off the crude monoacetate. Three g. of this crude leaflet-like product was recrystallized from 50 cc of benzene, 1 g. of the pure compound was recovered; m.p. 213°-215°C.

2.5.3   Synthesis of Fluorescein Diacetate[6,7]
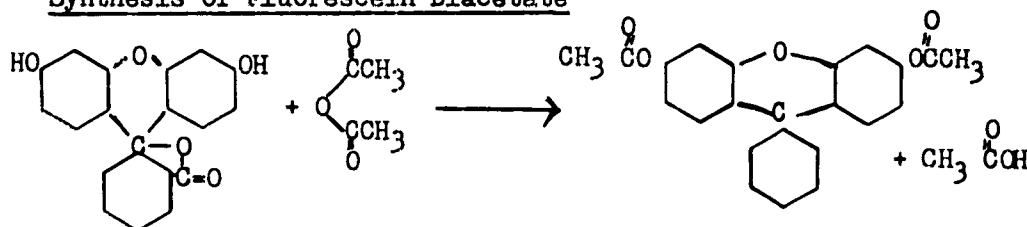
Procedure: In a 3-neck 250 ml. flask, place 20 g. (.06 moles) of fluorescein and 86.9 g. (.85 moles) of acetic anhydride; add two drops of H_2SO_4 and reflux the mixture for one hour. Then pour the mixture into H_2O (approximately 200 ml), filter, wash, and dry the precipitate.

The resulting diacetate (24.3 g.) was recrystallized from 650 ml. of acetone recovering 15 g. of pure compound; yield 61.2%; m.p. 206°-207°C.

2.5.4   Synthesis of p-Nitrothiophenol Acetate [8-12]
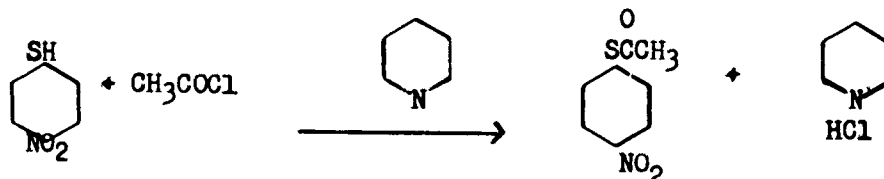
Procedure: In a 250 ml. beaker, dissolve 10 g. (.064 moles) of p-Nitrothiophenol* in 80 g. pyridine, and keep the solution cool in ice

* Kand K Laboratories, Jamaica, N.Y.

19

H_2O. Add dropwise 5.53 g. (.075 moles) of acetylchloride with stirring

to the p-Nitrothiophenol-pyridine solution (Note: keep the reaction cool

during the addition). After standing for 6 to 10 hours, the solution

is added to cold, 10% H_2SO_4' (approximately 550 ml), filtered, and washed

three times with 200 ml. portions of H_2O. 10.6 g. of crude product

was obtained and recrystallized from approximately 300 ml. of acetone.

2.5 g. of pure product was recovered; yield 19.7 %; m.p. 180-182°C.

2.6   Conclusions

The results obtained during this quarter indicate that the

enzymatic approach for the detection of microorganisms may be a feasible

approach. All the cultures tested, 12 bacterial cultures and one yeast

culture, were found to possess phenylacetate esterase and alkaline phos-

phatase. The data indicated that both phenylacetate esterase and alkaline

phosphatase may be universal enzymes, because they were present on all the

microorganisms tested for enzyme activity.

Also comparisons of phenylacetate esterase activity of a lyo-

philized preparation of Serratia marcescens with a non-lyophilized pre-

paration showed that lyophilization of the bacteria did not appreciably

decrease the enzyme activity. This conclusion is derived from the data

which shows that the amount of substrate hydrolized per cell of the lyo-

philized culture was ten times that hydrolyzed per cell of the non-lyo-

philized, in the first minute of reaction.

2.7   Proposed Effort for the Next Period

The proposed research to examine the possible use of microbial

enzymes for detection purposes will follow two courses. The first will be

the application of possible amplification techniques to increase the

20

sensitivity of enzyme assay techniques. The use of fluorescent substrates for amplifying the effects of esterase enzymes is one approach which will be explored. The measurement of fluorescent substrate hydrolysis depends on (1) observable differences between the fluorescence peaks of the esters and free dyes and (2) the solubility of the fluorescent substrates in water or other solvent compatible with the microbial enzyme system.

The assumption that all of the fluorescent substrates will meet these criteria may not be valid and therefore some of the promising fluorescent esters may have to be modified or not evaluated.

Another method which will be explored is the activation of papain by thiol groups. Dr. Warshowsky suggested that bacteria may have free sulfhydryl groups on their surfaces which would activate papain directly. This possibility will be investigated in the next quarter. The bacteria will also be tested for the presence of an enzyme which might hydrolyze p-nitrothiophenyl acetate, one of the esters synthesized during the first quarter. If this ester is hydrolized the reaction product (p-nitrothio-phenol) should activate papain.

The second area of investigation will be the resolution of the optimum conditions for the esterase and phosphatase assays. In this study the conditions which affect enzyme activity such as pH, temperature, ionic strength, substrates activations and chelating agents will be in-vestigated.

TABLE 1

Microscopic and Plate Counts of Microorganisms
Tested for Esterase Activity

Organism	Microorganisms per ml Suspension	
	Microscopic	Plate (viable)
Beggiotoa alba	5.6×10^9	4.0×10^9
Proteus vulgaris	2.0×10^9	1.8×10^9
*Shigella flexneri	1.1×10^9	
Staphylococcus aureus	1.6×10^9	1.2×10^9
Serratia marcescens	2.1×10^9	1.7×10^9
Saccharomyces cerevisiae	3.3×10^9	2.2×10^9
Azotobacter vinelandii	2.0×10^9	2.0×10^8
Bacillus globigii	5.1×10^9	4.0×10^9
Aeromonas hydrophila	12.0×10^9	9.0×10^9
Pseudomonas stutzeri	13.5×10^9	9.6×10^9
Bacillus pumilus	14.0×10^9	10.0×10^9
**Serratia marcescens	9.0×10^9	6.0×10^9
**Bacillus globigii	6.4×10^9	less than 10^6

* No plate counts were made.

** Lyophilized cultures obtained from Dr. Warshowsky, the Project Officer.

TABLE 2

Hydrolysis of Phenyl acetate by Microorganisms[**]

Organism	Microscopic Count	μ moles Ht/Organism in 10 min.	Molecules phenyl acetate hydrolyzed/ organism/min.
Beggiotoa alba	5.6×10^9	18.6×10^{-11}	3.1×10^5
Proteus vulgaris	2.0×10^9	17.0×10^{-11}	2.8×10^5
Shigella flexneri	1.1×10^9	32.0×10^{-11}	5.1×10^5
Staphylococcus aureus	1.6×10^9	5.6×10^{-11}	9.3×10^4
Serratia marcescens	2.1×10^9	8.6×10^{-11}	1.4×10^5
Saccharomyces cerevisiae	3.3×10^9	4.2×10^{-11}	7.0×10^4
Azotobacter vinelandii	2.0×10^9	11.5×10^{-11}	1.9×10^5
Bacillus globigii	5.1×10^9	12.0×10^{-10}	2.0×10^6
Aeromonas hydrophila	12.0×10^9	1.3×10^{-10}	2.2×10^5
Pseudomonas stutzeri	13.5×10^9	7.5×10^{-11}	1.2×10^5
Bacillus pumilus	14.0×10^9	9.5×10^{-11}	1.6×10^5
[*]Serratia marcescens	9.0×10^9	17.7×10^{-11}	2.9×10^5
[*]Bacillus globigii	6.4×10^9	0	0

[*] Lyophilized preparation obtained from Dr. Warshowsky, the Project Officer.

[**] Based on amount of ester hydrolyzed after ten minutes of enzyme reaction.

23

TABLE 3

Initial reaction rate for hydrolysis of Phenyl Acetate by Microorganisms[*]

Organism	Microscopic Count	Phenylacetate hydrolyzed/organism/min		
		μ Moles	Moles	Molecules
B. alba	5.6×10^9	0.67	12.0×10^{-17}	2.0×10^6
P. vulgaris	2.0×10^9	0.15	6.5×10^{-17}	1.1×10^6
S. flexneri	1.1×10^9	0.15	13.5×10^{-17}	2.2×10^6
S. aureus	1.6×10^9	0.03	1.9×10^{-17}	3.0×10^5
S. marcescens	2.1×10^9	0.01	5.0×10^{-18}	8.0×10^4
S. cerevisiae	3.3×10^9	0.02	6.0×10^{-18}	1.0×10^5
A. vinelandii	2.0×10^9	0.07	3.5×10^{-17}	5.7×10^5
B. globigii	5.1×10^9	0.70	13.5×10^{-17}	2.2×10^6
A. hydrophila	12.0×10^9	0.78	6.5×10^{-17}	1.1×10^6
P. stutzeri	13.5×10^9	0.20	1.4×10^{-17}	2.5×10^5
B. pumilus	14.0×10^9	0.58	4.1×10^{-17}	6.9×10^5
[**]S. marcescens	9.0×10^9	0.9	10.0×10^{-17}	1.67×10^6
[**]B. globigii	6.4×10^9	0.0	0.0	0.0

[*] Based on the first minute of reaction by the enzyme.

[**] Lyophilized preparations obtained from Dr. Warshowsky, the Project Officer. Bacillus globigii is apparently non-viable (see Table 1).

TABLE 4

Hydrolysis of p-nitrophenyl phosphate (p-NP) by Microorganisms

Organism	Microscopic Count	u Moles p-NP Hydrolyzed/ organism/30 min	Molecules p-NP hydrolyzed/ Organism/min
Serratia marcescens	2.2×10^9/cc	1.1×10^{-10}	6.0×10^4
S. cerevisiae (yeast)	4.4×10^8/cc	4.5×10^{-10}	2.4×10^5
Azotobacter vinelandii	2.2×10^8/cc	4.5×10^{-10}	2.4×10^5
Bacillus subtilis	5.2×10^9/cc	9.7×10^{-11}	5.3×10^4
Escherichia coli	7.3×10^9/cc	2.05×10^{-11}	1.1×10^4
Sarcina lutea	4.4×10^9/cc	1.37×10^{-10}	7.6×10^4
Bacillus globigii	5.5×10^9/cc	9.1×10^{-11}	5.0×10^4
Bacillus pumilus	13.5×10^9/cc	5.9×10^{-12}	3.2×10^3
Aeromonas hydrophila	12.9×10^9/cc	15.0×10^{-11}	8.0×10^4
Pseudominas stutzeri	14.5×10^9/cc	7.0×10^{-12}	3.6×10^3
Aerobacter cloacae	9.5×10^9/cc	4.2×10^{-11}	2.3×10^4
Aerobacter aerogenes	9.1×10^9/cc	5.5×10^{-12}	3.0×10^3

HYDROLYSIS OF PHENYLACETATE
BY MICROBIAL ESTERASE
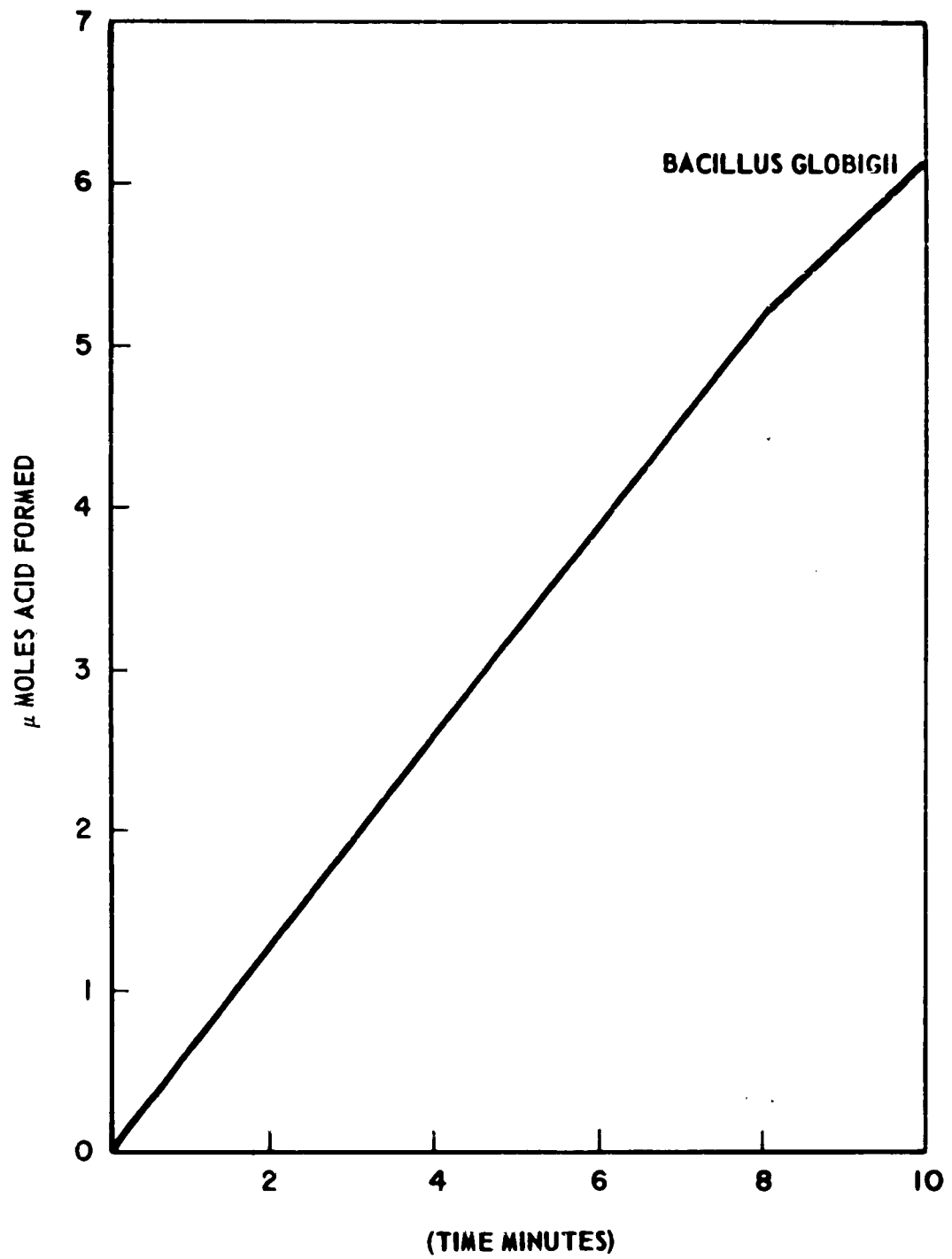
Figure 1

HYDROLYSIS OF PHENYLACETATE
BY MICROBIAL ESTERASE
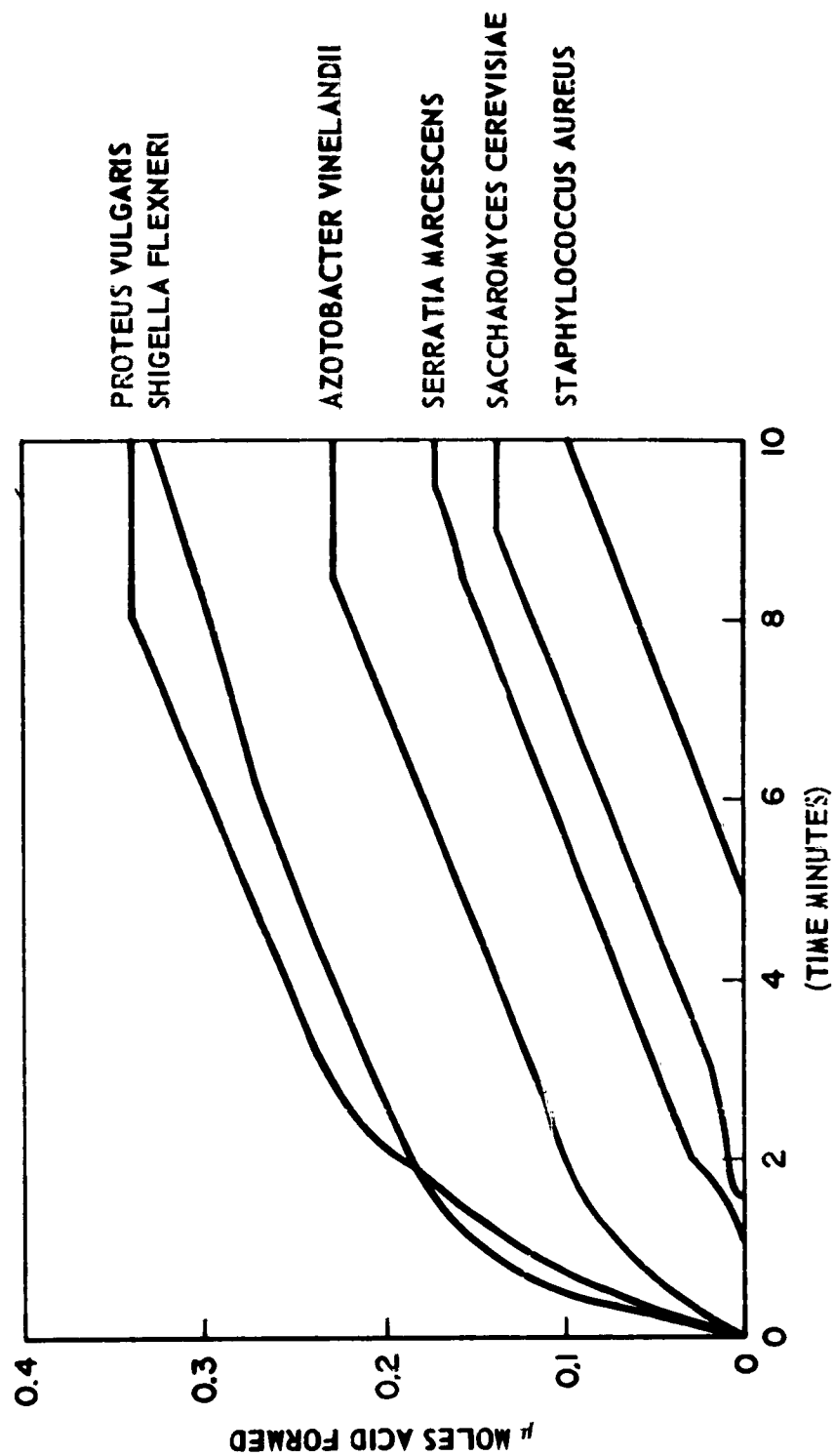
PROTEUS VULGARIS
SHIGELLA FLEXNERI

AZOTOBACTER VINELANDII

SERRATIA MARCESCENS

SACCHAROMYCES CEREVISIAE

STAPHYLOCOCCUS AUREUS

μ MOLES ACID FORMED

0.4   0.3   0.2   0.1   0

(TIME MINUTES)

0   2   4   6   8   10

Figure 2

HYDROLYSIS OF PHENYLACETATE
BY MICROBIAL ESTERASE

AEROMONAS HYDROPHILA

BACILLUS PUMILUS

BEGGIOTOA ALBA

PSEUDOMONAS STUTZERI

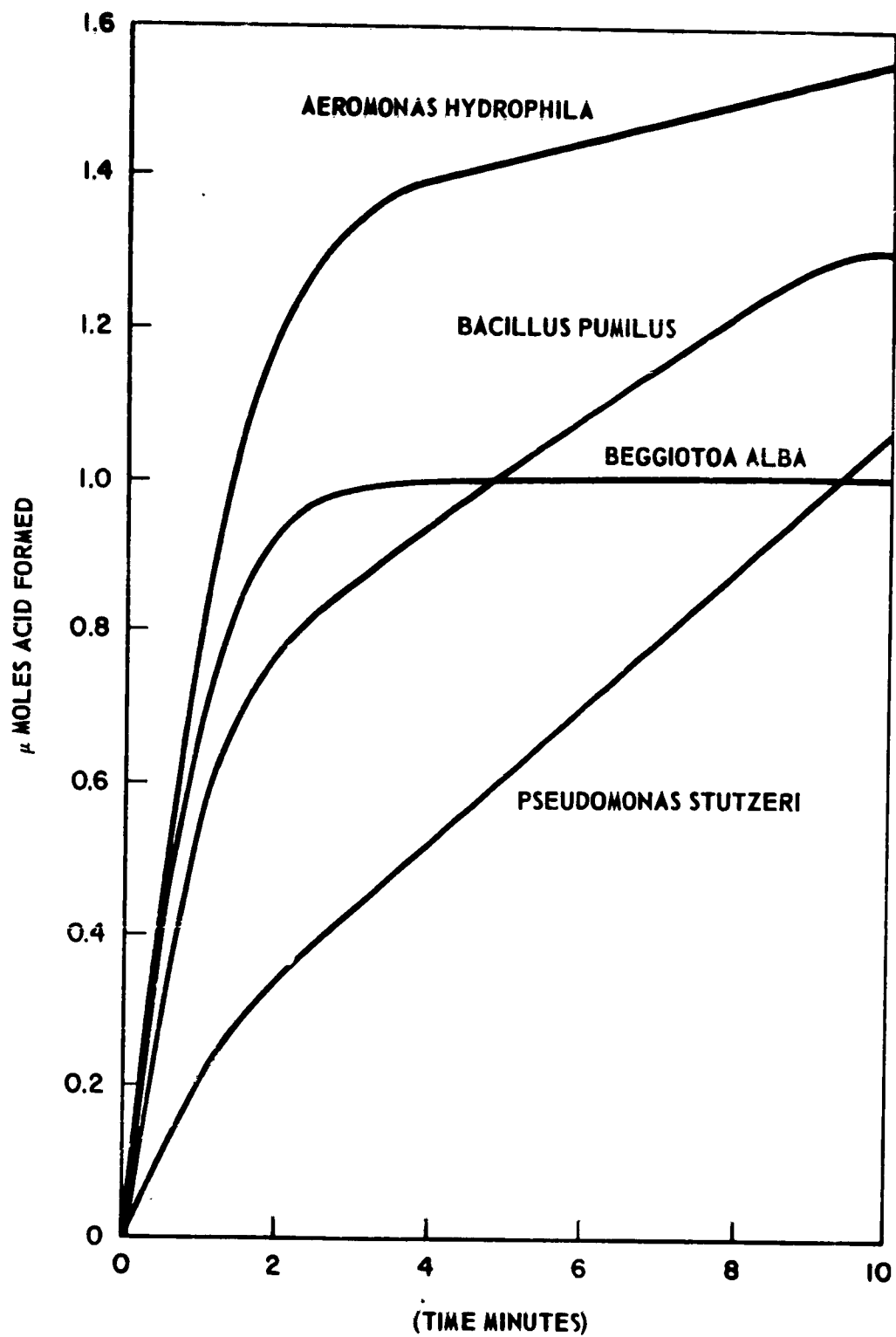μ MOLES ACID FORMED

(TIME MINUTES)

Figure 3

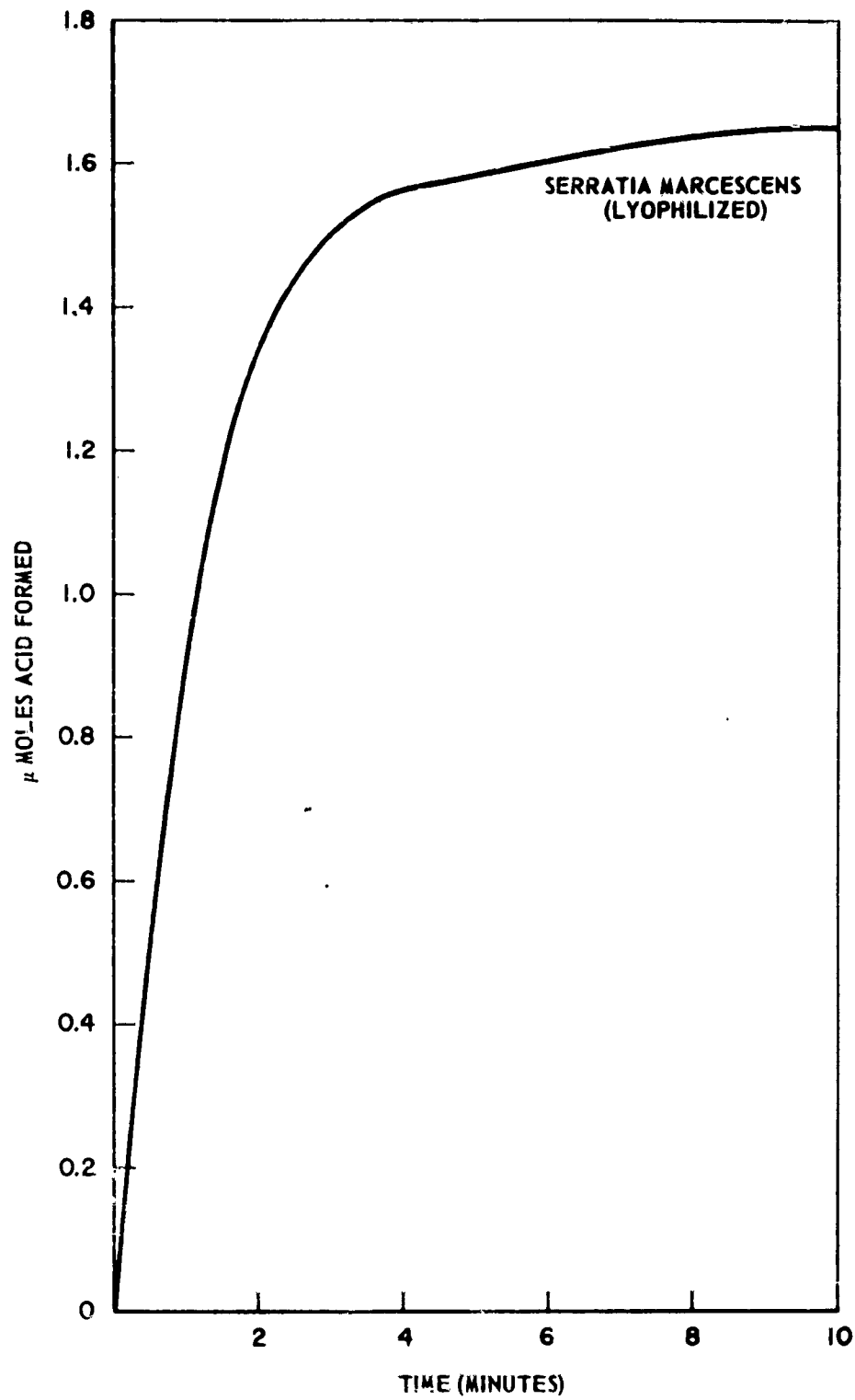HYDROLYSIS OF PHENYLACETATE
BY MICROBIAL ESTERASE

Figure 4

2.8   References

1.   Blanchard, G.C. Effects of lipopolysaccharide from **Escherichia coli** on some enzymes. PH.D dissertation, Syracuse University, Syracuse N.Y., 1961.

2.   Bessey, O.A., O.H. Lowry and M.J. Brock. "A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum." J. Biol. Chem. 164, 321, 1946.

3.   Rotman, B. Measurement of activity of single molecules of B-galactosidase. Proc. Nat. Acad. Sci. 47, 1981, 1961.

4.   Ghatak, N. and S. Dunn, J. Indian Chem. Soc. 6, 465-471, 1929.

5.   Beilstein XIX (722)

6.   van Liebig, H. J. fur Praktische Chemie 85, 267, 1912.

7.   Vogel, A. A Textbook of Practical Organic Chemistry Third ed. Wiley & Sons, New York, 1956, p. 987.

8.   Weygand, C. Organic Preparation, Interscience Publ. New York, 1945, p. 181.

9.   Malisoff, Chem. Rev. 7, 493-547, 1930.

10.   Jones and Tasher, J. Chem. Soc. 95, 1904, 1909.

11.   Ralston, J. Org. Chem. 4, 503, 1939.

12.   Wenzel and Reid, J. Am. Chem. Soc. 59, 1089, 1937.

13.   Kramer and Gamson, Army Chemical Corps Report No. 32-48; J. Org. Chem. 24, 1742-1747, 1959; J. Biol. Chem. 235, 1785, 1960.

APPENDIX

Outline of BW Detection Program

The over-all objective is to detect 1-100 bacteria or viruses per liter of aerosol in five minutes by employing biochemical principles. There are a number of new methods which could be applied to this problem including enzymatic amplification, chromophoric substrates, detection of bioelectric means, spectrophotometric means and immunological means. Obviously all these methods cannot be investigated with the funds available, thus a priority has been placed on the catalytic processes i.e. enzyme amplification, chromophoric substrates, cofactors and bioelectric methods. These methods offer greater potential for reaching the lower limits of sensitivity required for the detection of small numbers of bacteria or viruses. The problem is complicated by the fact that bacteria which are aerosolized become dormant and fail to grow rapidly. This lag period in the growth of the microorganism, however, is not associated with a decrease in metabolic state. (Hershey and Bronfenbrenner, J. Gen. Physiol. 21, 721, 1938) Therefore, the enzymatic method offers speed as well as sensitivity and specificity. The program for the first year on the enzymatic approach is divided in five phases as outlined below.

1. Screening for an ideal Enzyme

The objective of this phase of the work is to find a highly active enzyme which is common to all organisms grown under various conditions (environment and media) and stable to drying and storage. In this phase of the program Bacillus subtilis var. niger, Serratia marcescens, and the

bacteria listed in Table 1 of proposal P 223-294 will be evaluated for hydrolase enzymes such as esterases and phosphatases. It is fairly certain that these enzymes are common to most of the bacteria. If no one enzyme is universal, then it may be possible to use the enzyme common to a particular group of organisms for detection purposes. Once the ideal enzyme is selected the bacteria will be grown on several substrates to make certain that the enzyme is synthesized on all substrates (constitutive) rather than sythesized on only one type of substrate (adaptive). The bacteria will then be dried (air or lyophilized) and tested at various intervals of time for enzyme activity and viability. Since a rapid response is essential, the enzyme must be stable to dehydration and storage. Recent work in this country and Russia indicate that enzymes may be associated with viruses. As these viruses become available they will be screened for enzyme activity by the same procedures as are used for bacteria. The chances are that more specific enzyme will be found on viruses than on bacteria. This will mean that individual methods will be developed for viruses.

2. Determination of conditions for optimum enzyme activity

After the ideal enzyme has been selected, conditions for obtaining maximum enzyme activity such as pH, temperature, substrates, and additives (activators or chelating agents) will be investigated. The response time and the number of bacteria detectable under optimal conditions will be determined. Standard assay procedures based on the state of the art will be used and no effort will be employed at this time to explore new methods. The data obtained during this phase will allow projected estimates as to the amplification factors necessary for detection of each of the organisms in the allotted time.

3. Amplified Methods

There are two possible approaches which might be used to amplify the effects of an enzyme found on an organism; (a) direct enzyme measurement using decreased volume effect and (b) amplification of effect by multiple enzyme systems. As an example of the first approach the hydrolysis of an ester such as 6-hydroxyfluoran by a bacterial esterase would be examined initially on the large scale (10^{12} bacteria per ml) by the spectrophotofluorimetric procedure. The sensitivity can be increased by a factor of 10^{12} by employing microscopic techniques where the volume is decreased from cm^3 to μ^3. With these procedures one instead of 10^{12} bacteria would be detectable. Boris Rotman (Proc. Nat. Acad. Sci. 47, 1981, 1961) used this microscopic procedure for measuring the activity of a single molecule of an enzyme called β-galactosidase. Because bacteria contain many molecules of most enzymes, more rapid detection is possible than would be observed for 1 enzyme molecule. β-galactosidase, unfortunately, is an adaptive enzyme, but the general principle might be applied to other enzymes.

The second approach, amplification by multiple enzymes, uses bacterial enzymes for hydrolyzing specific substrates to products. The products then activate other enzyme systems. While several enzyme couples might be tried, the system which looks most promising is the bacterial esterase amplified by papain. The bacterial enzyme hydrolyzes thiol esters to liberate sulfhydryl groups which activate papain. Papain then hydrolyzes specific substrates and liberates more sulfhydryl groups which activate

more papain. An autocatalytic system like this should form enough product to be rapidly detected, especially if the hydrolysis product is something like p-nitrophenol which is easily measured spectrophotometrically.

The possibility that free sulfhydryl groups on the surface of bacteria would activate papain will be explored. If a system such as this were feasible then it might be possible to use some flow system where bacteria collected on a membrane would be constantly bathed in a solution containing the thiol esters and papain. Hydrolysis of the ester would then activate papain. Activation of papain by other reducing agents such as hydroquinone should also be investigated. It has already been demonstrated that hydroquinone esters are hydrolyzed by bacteria. Thus studies to determine if papain is activated would result in a possible detection system.

4. Use of Chromophoric Substrates and Bioelectric Methods for Rapid Readout of Responses

Many chromophoric compounds such as 2,6 dichloroindophenol, phenolphthalein, p-nitrophenol, fluorescein, 6-hydroxyfluoran, other dyes and indicators can be used in the synthesis of substrates for enzymes. While some of these substrates may be used in the quantitation work in phase I through III, attempts will be made in phase IV to simplify the techniques for rapid visual readout, with as little instrumentation as possible. It may be possible to use some chromophoric substrate to measure papain activation by the sulfhydryl groups present on the bacterial surface. Such a technique would be extremely sensitive.

There are several chromophoric compounds, such as phenolphthalein, fluorescein, 6-hydroxyfluoran, phenolsulfonophthalain, p-nitrophenol and 2,6 dichloroindophenol, which can be conjugated into esters, ethers and

glycosides. These conjugated compounds serve as enzyme substrates and when they are hydrolyzed they give some observable change (i.e. change in color or color production) Efforts will be made to purchase these chromophoric derivatives. Some of the chromophoric substrates may have to be synthesized. The particular substrate prepared however will depend on the results obtained in the studies on the universal enzyme.

A brief study on new analysis techniques for detecting bacteria or products formed by enzymic hydrolysis of substrate will also be carried out in this phase. This will employ existing set ups and know how. Studies based on coulometric titration, pH stat measurements of changes in pH or Eh, and biochemical fuel cell measurements of redox couples are now being carried out at Melpar on other projects and may be useful in rapid detection systems. As these techniques appear to apply, they will be tested. Sensitive coulometric or amperometric techniques may be of value not only for measuring substrate hydrolysis but also for following direct reaction with the bacteria. For example, it may be possible to brominate the phenolic groups or to iodinate certain amino acids (i.e. tyrosine) or polysaccharides that occur in bacterial cell walls. These titrations are easily followed by measuring the amount of bromine or iodine generated or by following the amount of the elements utilized in the reaction.

The use of the pH stat to follow pH changes or changes in the oxidation reduction potentials (Eh) caused by the activity of bacterial enzymes on particular substrates is also a sensitive and quantitative means of measuring bacterial numbers. With the latter technique, the bacterial response can be continuously monitored with a recorder. If

35

these techniques appear to work with large concentrations of organisms, an effort will be exerted to increase the sensitivity. One of the techniques that would be most useful for increasing sensitivity would be to increase the electrode size by the use of thin films.

5. Instrumentation

As the feasibility of the various methods are tested, recommendations will be made for improving and/or implementing the best method.

Future Considerations

The successful conclusion of the first years effort will pose many new questions which must be answered before the new method can be considered for adoption. Some of the questions which will have to be considered are: (1) What are the effects of aerosolization, exposure and dehydration on enzyme activity and time of response? (2) What are the best ways to bring bacteria and substrate together? (3) What are the best kinds of readout systems? and (4) What are the best kinds of instrumentation? These are the usual situations which arise after one has established the feasibility of a new detection principle.